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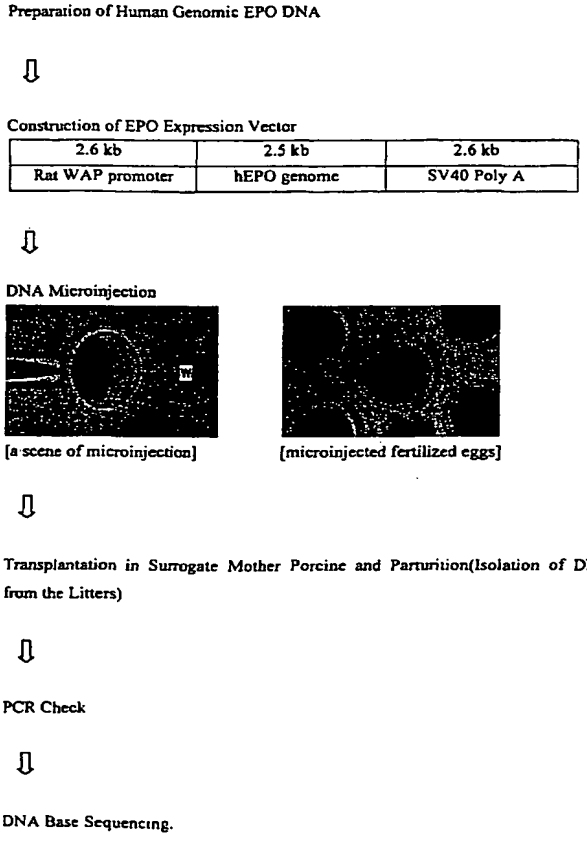
[Continued on next page]

(54) Title: THE PRODUCTION METHOD OF TRANSGENIC PORCINE PRODUCING HUMAN ERYTHROPOIETIN AND THE TRANSGENIC PORCINE



(57) **Abstract:** Disclosed are transgenic porcine capable of secreting human erythropoietin (EPO) in their milk and the preparation thereof. For the preparation of the transgenic porcine, a 2.6 kb WAP promoter from the mammary gland of a rat is first amplified by PCR. Along with this PCR product, the human EPO genome DNA fragment and an SV40 poly A DNA fragment are used to construct an expression vector. Separately, PMSG and human chorionic gonadotrophic (hCG) hormone are administered into porcine by intramuscular injection to induce porcine to ovulate excessively and the porcine were led to natural mating. From the porcine, the fertilized eggs in the first cell differentiation period are collected. Next, the expression vector is injected into male pronuclei which are immediately transplanted in surrogate mother porcine. The surrogate mother porcine are allowed to give birth to litters. Therefore, the present invention can produce the expensive medicine human EPO at low costs on a large scale, giving a contribution to the improving of human health.

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THE PRODUCTION METHOD OF TRANSGENIC PORCINE PRODUCING HUMAN ERYTHROPOIETIN AND THE TRANSGENIC PORCINE

TECHNICAL FIELD

The present invention relates to transgenic porcine that are able to produce human erythropoietin useful as a medicine. More particularly, the present invention relates to transgenic porcine that are able to secrete human erythropoietin in their milk, thereby producing the useful medicine at a low cost on a large scale with stability. Also, the present invention is concerned with a method for preparing such transgenic porcine.

BACKGROUD OF PRIOR ART

With an average life span of 120 days, human erythrocytes are generally destroyed at a level of one hundred-twentieth of their total number everyday in the reticuloendothelial system. However, they show homeostasis because they are newly produced equally every day (Guyton, Textbook of Medical Physiology, pp56-60, W. B. Saunders Co., Philadelphia (1976)).

Erythrocytes are produced in the bone marrow through maturation and differentiation of erythroblasts during which the hormone EPO serves as a factor to stimulate the differentiation of less-differentiated cells into erythrocytes (Guyton, *supra*).

In the 1950s, EPO was found by observing the fact that a large amount of $_{59}Fe$ was incorporated into newly forming erythrocytes when sera of anemic animals were introduced into normal animals (Borsook, et al., Blood, 9, 734(1954)). A lack of oxygen or a shortage of erythrocytes owing to, for example, hemorrhage, or an increase of the number of anemic cells stimulates cells in the kidney of adults to synthesize and secrete increased amounts of erythropoietin into the bloodstream. This hormonal glycoprotein plays an important role in the control of erythropoiesis and the maintenance of the number

of erythrocytes in blood (Carnot et al., Comot. Rend. 143, 384 (1906); Kranz. S. B., Blood 77, 419(1991); Goldwasser, E., et al., in Peptide Growth Factors and their Receptors I, Sporn, M. B. and A. B. Roberts, eds., Springer-Verlag, Berlin, p. 747 (1990)).

As well known in the art, natural type EPO, which is responsible for the control of erythropoiesis, is secreted from the liver in fetuses. The secretion function for the EPO begins to move into the kidney at 120-140 days after the conception and the transferring of the secretion function is completed 40 days after the parturition. In adults, the kidney produces most of EPO while the liver is responsible for the secretion of EPO at a level of 10% of the total amount secreted. In addition, a little amount of EPO is also known to be secreted in macrophages of the bone marrow.

EPO is maintained at a level of 15-30 mU per ml of blood or at a level of 0.01 mM in blood (Garcia, J. F., Lab. Clin. Mde. 99, 624-635 (1982)). Higher levels of EPO in blood are measured from the patients suffering from aplastic anemia than from normal persons, so that the blood and/or urine of the patients are utilized to produce EPO (White, et al., Rec. Prog. Horm. Res. 16, 219 (196); Espada, et al., Biochem. Med. 3, 475 (1970); Fisher, pharmacol. Rev. 24, 459 (1972)).

As mentioned early, EPO is a glycoprotein with a molecular weight of about 30 kD, in which sugar chains are attached in N-glycosidic linkage to the 24th, the 38th and the 83rd amino acid residues and a sugar chain is attached in O-glycosidic linkage to the 126th amino acid residue (P. S. E. B. M. 216, 358-369 (1997)). Conventionally, EPO was produced in animal cells by a recombinant technique, but at low amounts. In addition, the recombinant EPO suffers from the problems of being not identical in physiological functions to and of being poorer than natural type EPO.

EPO is very useful for the clinical treatment of anemic diseases, especially renal anemia and it is preferable that this therapeutic is prepared from human-derived materials owing to antigenicity. As mentioned early, EPO can be obtained by taking advantage of the blood or urine from patients suffering from

aplastic anemia. However, the amount of obtainable EPO from the patients, although being blood rich in EPO, is extremely limited.

From sera of sheep, EPO can be recovered in a stable water soluble form with a satisfactory titer, but this animal EPO includes the problem that it might act as an antigen to the human body.

Biotechnology Co. Ltd., Cuba, took advantage of human erythropoietin (hEPO) cDNA to create a transgenic rabbit from which hEPO is secreted through its mammary gland. Likewise, Kuopioeogkr, Finland, was reported to have created a transgenic mouse capable of secreting hEPO via its mammary gland. However, there have been found no reports which disclose transgenic porcine capable of secreting hEPO. Korean Pat. Publication No. 93-5917 describes that an hEPO gene is cloned and expressed in mammalian or insect cells. Not only is the EPO expressed only at a small amount in this process, but also glycosylation does not occur accurately so that the EPO is degraded rapidly in the body. In Korean Pat. Appl'n No. 94-12082, an expression vector carrying a modified recombinant hEPO (rhEPO) is used to transform the animal cell COS-7 (ATCC CRL 1651, African green monkey kidney cell) into one which is able to produce rhEPO. This method, however, is unsuitable for large-scale production because of requiring continual transformation.

Korean Pat. No. 184778 discloses a method of producing rhEPO with stability and efficiency, which takes advantage of a permanent strain cell transfected by an expression vector carrying an hEPO gene. This patent is quite different from the present invention pertaining to the production of rhEPO in porcine milk.

DISCLOSURE OF THE INVENTION

Leading to the present invention, the intensive and thorough research on the production of human EPO, repeated by the present invention, resulted in the finding that a WAP promoter, in combination with SV40 Poly A, is very useful to incorporate a human EPO gene into the genomic DNA of porcine and the

recombinant expression vector can be used to create transgenic porcine which can secrete human EPO in their milk with stability.

Therefore, it is an object of the present invention to overcome the above problems encountered in prior arts and to provide transgenic porcine that are able to secrete human EPO in their milk.

It is another object of the present invention to provide a method for preparing transgenic porcine capable of producing human EPO at low costs with stability.

In accordance with an embodiment of the present invention, there are provided transgenic porcine (named "Saerome") capable of secreting human EPO in their milk with stability.

In accordance with another embodiment of the present invention, there is provided a method for preparing transgenic porcine capable of secreting human EPO in their milk, comprising the steps of: amplifying a 2.6 kb WAP promoter from the mammary gland of a rat by a polymerase chain reaction; constructing an expression vector comprising a human erythropoietin genome DNA fragment and an SV40 poly A DNA fragment; administering PMSG and human chorionic gonadotrophic (hCG) hormone into porcine by intramuscular injection to induce porcine to ovulate excessively; determining the porcine as to their oestrus and leading them to natural mating; collecting the fertilized eggs in the first cell differentiation period from the porcine; injecting the expression vector into male pronuclei and immediately transplanting them in surrogate mother porcine; allowing the surrogate mother porcine to give birth to litters; and identifying the incorporation of the base sequence of the Sequence List 1 into the genomic DNA of the progeny.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a schematic process flow showing the preparation of transgenic porcine which are able to secrete human EPO in their milk;

Fig. 2 shows the incorporation of human EPO gene into the genomic DNA of porcine through a polymerase chain reaction; and

Fig. 3 is a base sequence for a human EPO cDNA incorporated into the genomic DNA of porcine.

BEST MODES FOR CARRYING OUT THE INVENTION

A detail description will be given of a transgenic porcine capable of producing hEPO in its milk, below, in conjunction with the drawings. Before the present transgenic porcine capable of producing hEPO and preparation method thereof are disclosed or described, it is to be understood that explanation of well-known functions or structures might be eliminated if it is judged to make unclear the substance of the present invention. Also, it must be noted that the terminology used therein is defined with the purpose of describing particular embodiments only, but not limiting, and may be changed in its definition depending on the intention or usage of users. Therefore, it should be defined on the basis of the through-context of the present invention.

With reference to Fig. 1, there is schematically shown the entire procedure that allows the production of transgenic porcine capable of secreting hEPO in their milk. As a material to prepare a recombinant human EPO gene, we obtained a human genomic DNA fragment comprising an EPO gene from Prof. Kim. J. H., of the department of animal husbandry, Korean National KyoungSang University. Using a polymerase chain reaction (PCR), a 2.6 kb WAP promoter was amplified from a mammary gland gene of a rat, and the PCR product was cloned. Along with an SV40 poly A gene and an hEPO gene, this promoter was used to construct a recombinant expression vector, which would serve as a DNA donor, as shown in Table 1, below.

TABLE 1
EPO Expression Vector

DNAs	Rat WAP promoter	hEPO gene	SV40 Poly A
Size	2.6 kb	2.5 kb	2.6 kb

Porcine were allowed to ovulate excessively by the intramuscular injection of P.M.S.G (eCG) hormone, which is a superovulation-inducing hormone, and human chorionic gonadotrophic (hCG) hormone. After the porcine were determined as to their oestrus and led to natural mating, the fertilized eggs in the first cell differentiation period were collected. The above expression vector was injected into male pronuclei which were immediately transplanted in surrogate mother porcine. One of the litters delivered from the surrogate mother porcine was found to carry DNA fragments encoding human EPO as measured from its tail, blood and sperm by PCR. This result is given as shown in Fig. 2.

Given in the following Table 2 are the primer sequences which were used for the PCR for the determination as to whether the litters had the DNA fragments of interest.

TABLE 2

Primers	Sequences	Expected Sizes
Hepo-304	F 5'- CGA GAA TAT CAC GGT AGA ACC -3' R 5'- CTC ATT CAA GCT GCA GTG TTC -3'	304 bp
Hepo-567	F 5'- AAG TGG TGC ATG GTG GTA GTC -3' R 5'- TTA CAG AAA GGG CAA GCA GAA -3'	567 bp

Blood was taken from the EPO transgenic porcine and analyzed for erythrocyte properties. The results are given in Table 3, below.

TABLE 3

	No. of Erythrocytes ($x10^6$/ul)	Vol. Of Erythrocytes (%)
Control	4.63(100)	66.5(100)
Transformed	5.25(113)	78.3(118)

Electrophoresis of PCR products obtained from various copies of the genomic DNA of the litter delivered through the surrogate mother porcine gave information incorporated into the genomic DNA. Base sequencing analysis confirmed the incorporation, identifying the cDNA as having the base sequence shown in the following Base Sequence List. We named the resulting transgenic porcine "Saerome".

[SEQUENCE LIST]

Sequence No.: 1
Length of Sequence: 582
Type of Sequence: Nucleic Acids
Number of Strand: Double Strand
Topology: Linear
Type of Molecules: cDNA
Origin
EPO cDNA obtained from human liver DNA
Characteristics of Sequence
Mark representing a Characteristic: sig peptide
Position located: 1-81
Mark representing a Characteristic: mat peptide
Position located: 82-582

Mark representing a Characteristic: terminator

Position located: 580-582

[SEQUENCE 1]

```
ATG GGG GTG CAC GAA TGT CCT GCC TGG CTG TGG CTT CTC CTG TCC 45
Met Gly Val His Glu Cys Pro Ala Trp Leu Trp Leu Leu Leu Ser
-27                      -20
CTG CTG TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA  90
Leu Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Prp Pro
        -10                                      +1
CGC CTC ATC TGT GAC AGC CGA GTC CTG GAG AGG TAC CTC TTG GAG  135
Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu
                         10
GCC AAG GAG GCC GAG AAT ATC ACG ACG GGC TGT GCT GAA CAC TGC  180
Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys
    20                                   30
AGC TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC  225
Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
                         40
TAT GCC TGG AAG AGG ATG GAG GTC GGG CAG CAG GCC GTA GAA GTC  270
Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val
     50                                  60
TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT GTC CTG CGG GGC CAG  315
Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln
                         70
GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG CAG CTG  360
Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu
     80                                  90
CAT GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG  405
His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu
                        100
```

```
CTT CGG GCT CTG GGA GCC CAG AAG GAA GCC ATC TCC CCT CCA GAT   450
Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp
     110                                             120
GCG GCC TCA GCT GCT CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC   495
Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe
                        130
CGC AAA CTC TTC CGA GTC TAC TCC AAT TTC CTC CGG GGA AAG CTG   540
Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu
     140                                             150
AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG GAC AGA TGA       582
Lys Leu Tyr Thr Gly Gly Ala Cys Arg Thr Gly Asp Arg
                        160
```

As described hereinbefore, the present invention provides transgenic porcine capable of secreting human EPO in their milk, so that the expensive useful medicine can be produced at a low cost with stability on a large scale, thereby giving a contribution to the improving of human health.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

CLAIMS

1. A method for preparing transgenic porcine capable of secreting human erythropoietin in their milk, comprising the steps of:

amplifying a 2.6 kb WAP promoter from the mammary gland of a rat by a polymerase chain reaction;

constructing an expression vector comprising a human erythropoietin gemome DNA fragment, and an SV40 poly A DNA fragment;

administering PMSG and human chorionic gonadotrophic (hCG) hormone into porcine by intramuscular injection to induce porcine to ovulate excessively;

determining the porcine as to their oestrus and leading them to natural mating;

collecting the fertilized eggs in the first cell differentiation period from the porcine;

injecting the expression vector into male pronuclei and immediatcly transplanting them in surrogate mother porcine;

allowing the surrogate mother porcine to give birth to litters; and

identifying the incorporation of the base sequence of the Sequence List 1 into the genomic DNA of the progeny.

2. Transgenic porcine capable of producing human erythropoietin, prepared according to the method of claim 1.

3. The method as set forth in claim 1, wherein the expression vector comprises a 2.6 kb rat WAP promoter, a 2.5 kb hEPO and a 2.6 kb SV40 Poly A.

4. The method as set forth in claim 1, wherein the human erythropoietin cDNA comprises the base sequence shown in Fig. 3.

5. The transgenic porcine as set forth in claim 2, wherein the sperm DNA of the porcine comprises a gene coding for WAP-EPO.

6. The transgenic porcine as set forth in claim 2, wherein the human erythropoietin is WAP-EPO.

6'. The transgenic porcine as set forth in claim 2, wherein the human erythropoietin is produced in a form of WAP-EPO.

7. The transgenic porcine as set forth in claim 2, wherein the transgenic porcine is "Saerome".

8. The transgenic porcine as set forth in claim 2, wherein litters of the transgenic porcine have a WAP-EPO DNA.

9. The transgenic porcine as set forth in any of claims 1 to 8, wherein the produced erythropoietin can be readily used as a medicine.

[FIG. 1]

Preparation of Human Genomic EPO DNA

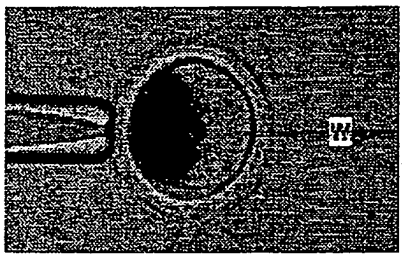
⇩

Construction of EPO Expression Vector

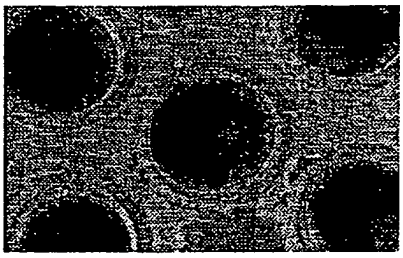
2.6 kb	2.5 kb	2.6 kb
Rat WAP promoter	hEPO genome	SV40 Poly A

⇩

DNA Microinjection



[a scene of microinjection]



[microinjected fertilized eggs]

⇩

Transplantation in Surrogate Mother Porcine and Parturition(Isolation of DNA from the Litters)

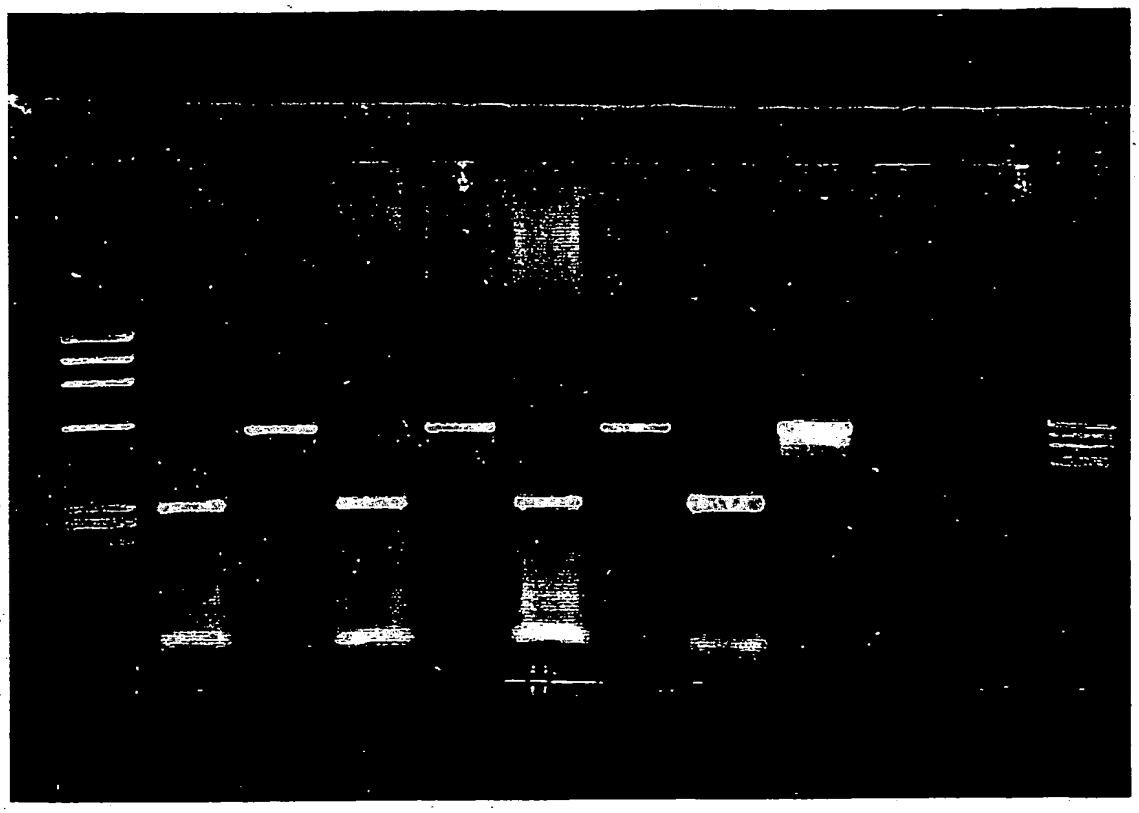
⇩

PCR Check

⇩

DNA Base Sequencing.

[FIG. 2]



[FIG. 3]

```
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-27                         -20
CTG CTG TCG CTC CCT CTG GGC CTC CCA GTC CTG GGC GCC CCA CCA   90
Leu Leu Ser Leu Pro Leu Gly Leu Pro Val Leu Gly Ala Prp Pro
        -10                                         +1
CGC CTC ATC TGT GAC AGC CGA GTC CTG GAG AGG TAC CTC TTG GAG  135
Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu Leu Glu
                            10
GCC AAG GAG GCC GAG AAT ATC ACG ACG GGC TGT GCT GAA CAC TGC  180
Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His Cys
    20                                      30
AGC TTG AAT GAG AAT ATC ACT GTC CCA GAC ACC AAA GTT AAT TTC  225
Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
                        40
TAT GCC TGG AAG AGG ATG GAG GTC GGG CAG CAG GCC GTA GAA GTC  270
Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val
    50                                      60
TGG CAG GGC CTG GCC CTG CTG TCG GAA GCT GTC CTG CGG GGC CAG  315
Trp Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln
                        70
GCC CTG TTG GTC AAC TCT TCC CAG CCG TGG GAG CCC CTG CAG CTG  360
Ala Leu Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu
    80                                      90
CAT GTG GAT AAA GCC GTC AGT GGC CTT CGC AGC CTC ACC ACT CTG  405
His Val Asp Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu
                        100
CTT CGG GCT CTG GGA GCC CAG AAG GAA GCC ATC TCC CCT CCA GAT  450
Leu Arg Ala Leu Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp
    110                                     120
GCG GCC TCA GCT GCT CCA CTC CGA ACA ATC ACT GCT GAC ACT TTC  495
Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe
                        130
CGC AAA CTC TTC CGA GTC TAC TCC AAT TTC CTC CGG GGA AAG CTG  540

Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu Arg Gly Lys Leu
    140                                     150
AAG CTG TAC ACA GGG GAG GCC TGC AGG ACA GGG GAC AGA TGA      582
Lys Leu Tyr Thr Gly Gly Ala Cys Arg Thr Gly Asp Arg
                        160
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Sequence Listing

<110> Republic of Korea(Management:Rural Development Administration)
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MIN, Kwan-Sik
YANG, Bo-Seok
IM, Gi-Sun
LEE, Yun-Keun
LEE, Chnag-Hyun
KIM, Jin-Hoei

<120> THE PROCUCTION METHOD OF TRANSGENIC PORCINE PRODUCING HUMAN ERYTH ROPOIETIN AND THE TRANSGENIC PORCINE

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             20                  25                  30

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         35                  40                  45

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     50                  55                  60

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Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe Tyr Ala Trp Lys Arg
 65                  70                  75                  80

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                 85                  90                  95

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             100                 105                 110

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Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
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115 120 125

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130 135 140

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Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
145 150 155 160

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165 170 175

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35 40 45

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50 55 60

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Met Glu Val Gly Gln Gln Ala Val Glu Val Trp Gln Gly Leu Ala Leu
85 90 95

Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu Leu Val Asn Ser Ser
100 105 110

Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp Lys Ala Val Ser Gly
115 120 125

Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu Gly Ala Gln Lys Glu
130 135 140

Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala Pro Leu Arg Thr Ile
145 150 155 160

Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val Tyr Ser Asn Phe Leu
165 170 175

Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala Cys Arg Thr Gly Asp
180 185 190

Arg

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/00675

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
C12N 5/00

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

[redacted]

Electronic data base consulted during the international search (name of data base and, where practicable, search trerms used)
NCBI, pubmed, IBM patent database, USPTO patent database "Erythropoietin, transgenic"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5955422 A (Kirin-Amgen, Inc) 21 Sept, 1999 (21. 09.199)	1-9
A	Proc. Natl. Acad. Sci. USA,1990, 87:5178-5182.	1-9
A	Mol. Biol. Med.,1989, 5:255-261.	1-9
A	Transgenic Res, 1997, 6(1):75-84	1-9
A	DNA Cell Biol, 1999, 18(11):845-	1-9
A	Transgenic Res, 1998, 7 (4):311-7	1-9
A	Eur J. Biochem 1997, 245(2):482-9	1-9
A	Blood 1995, 85(10);2735-41	1-9
A	Biol Res 1995, 28(2);141-53	1-9

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
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"P" document published prior to the international filing date but later than the priority date claimed
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"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 DECEMBER 2000 (12.12.2000)	13 DECEMBER 2000 (13.12.2000)

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